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## **New Wistar Kyoto and Spontaneously Hypertensive rat transgenic models with ubiquitous expression of green fluorescent protein**

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## Abstract

The Wistar Kyoto rat (WKY) and the Spontaneously Hypertensive Rat (SHR) inbred strains are well-established models for human crescentic glomerulonephritis (CRGN) and metabolic syndrome respectively. Novel transgenic (Tg) strains add research opportunities and increase scientific value to well-established rat models. We have created two novel Tg strains using Sleeping Beauty transposon germline transgenesis, ubiquitously expressing green fluorescent protein (GFP) under the rat elongation factor 1 alpha (rEF1a) promoter on the WKY and SHR genetic backgrounds. The Sleeping Beauty system functioned with high transgenesis efficiency in our hands: 75% of the new rats born after embryo microinjections were transgene positive. By ligation-mediated PCR, we located the genome integration sites, confirming no exonic disruption and defining single or low copy number of the transgenes in the new WKY-GFP and SHR-GFP Tg lines. We report GFP bright expression in embryos, tissues and organs in both lines and show preliminary in vitro and in vivo imaging data that show the utility of the new GFP-expressing lines for adoptive transfer, transplantation and fate mapping studies of CRGN, metabolic syndrome and other traits for which these strains have been extensively studied over the past four decades.

## Introduction

The rat has long been an important physiological model of complex human disease (Aitman et al., 2008; Iannaccone and Jacob, 2009). Two well-studied strains, both originating from Wistar stock, are the Wistar Kyoto (WKY) and Spontaneously Hypertensive (SHR) inbred rats (Kurtz and Morris, 1987; Okamoto and Aoki, 1963). The Wistar Kyoto (WKY) rat has been studied extensively as a model of crescentic glomerulonephritis (Aitman et al., 2006; Atanur et al., 2013; Behmoaras et al., 2008; Behmoaras et al., 2015; D'Souza et al., 2013; Kanno et al., 1998; Sado et al., 1984; Smith et al., 2007), as a control for the SHR strain and as a model of depression and behavioural abnormalities (Will et al., 2003). The SHR strain has been widely used as a model of the human hypertension and metabolic syndrome as well as a large number of other pathophysiological phenotypes including, cardiac hypertrophy and failure, insulin resistance and defects in lipid metabolism (Aitman et al., 1999; Aitman et al., 1997; Lepretre et al., 2004; Pravenec et al., 2008; Yamori and Okamoto, 1974).

Both inbred strains are well characterised, with 199 and 197 quantitative trait loci (QTLs) mapped respectively in WKY and SHR (Shimoyama et al., 2015), and whole genome sequences of both strains now publically available (Aitman et al., 2008; Atanur et al., 2010; Atanur et al., 2013; Hubner et al., 2005; Shimoyama et al., 2015). With the advent of gene targeting technologies that are suited to use in rats, including Zinc Finger Nucleases (ZFNs) (Geurts et al., 2009), Transcription activator-like effector nucleases (TALENs) (Tesson et al., 2011), Clustered Regularly Interspaced Short Palindromic Repeats (CRISPRs) (Shen et al., 2013) and Sleeping Beauty (SB) transposon system (Ivics et al., 2009; Kitada et al., 2007; Mates, 2011),

increasing numbers of new transgenic (Tg), knockout and knock-in rat models have been created to elucidate the functional basis of disease phenotypes (Jacob et al., 2010)

Fluorescent imaging for in vitro and in vivo analysis of biological processes combined with GFP Tg models are valuable reagents for translational research. Tg models reporting ubiquitous enhanced green fluorescent protein (GFP) have been described in inbred rats mostly under the control of the chimeric CAG (cytomegalovirus enhancer, chicken  $\beta$ -actin enhancer-promoter, and intronic sequences from rabbit  $\beta$ -globin) promoter, using classical transgenesis (Hakamata et al., 2001), lentivirus integrations (Michalkiewicz et al., 2007) and Sleeping Beauty (Katter et al., 2013). However, there are shortcomings in some of these approaches. Classical transgenesis (Charreau et al., 1996; Mullins et al., 1990) has low efficiency and is likely to insert concatemer transgene copies in the genome. This can predispose to gene silencing and a high frequency of mosaic founders (Bishop and Smith, 1989; Garrick et al., 1998; Whitelaw et al., 1993). Lentivirus integrations have high efficiency, but also have drawbacks including triggering of transgene silencing by epigenetic regulation and production of mosaic founders. As well as limitations in transgene size, lentiviruses can cause embryo toxicity due to preferential transgene insertion in endogenous genes (Ellis, 2005; Hofmann et al., 2006; Lois et al., 2002; Wolf and Goff, 2009).

This paper describes the creation of two new ubiquitously expressing GFP models in WKY and SHR inbred rat lines by combining a highly efficient transgenic system and a strong mammalian endogenous promoter. We took advantage of the Sleeping

Beauty (SB) transposon system that randomly integrates single copies or low copy number of a gene of interest (Ivics et al., 2014; Mates, 2011). We opted for the rat Elongation Factor 1 alpha (rEF1a) promoter (Kim et al., 1990; Mizushima and Nagata, 1990). The rEF1a gene encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome (Sasikumar et al., 2012) and its promoter has been successfully used in gene therapy studies as a non-viral alternative to the cytomegalovirus promoter (Gill et al., 2001; Serafini et al., 2004; Zheng and Baum, 2005). We report GFP expression in embryos, tissues, *ex vivo* cell cultures and in an *in vivo* imaging model of bone marrow transplantation to validate these lines as useful tools for translational research.

## Materials and methods

All animals were housed in individually ventilated cages. All procedures were carried out according to the institutional guidelines for the care and use of experimental animals and the ARRIVE guidelines. Animal studies were approved by the UK Home Office.

### Generation of ubiquitous GFP WKY and SHR rats using Sleeping Beauty transgenesis.

*Preparation of the Transposon donor plasmid and microinjection:* Schematic of plasmid is shown in Figure 1A. The CAGGS promoter in pSB IR-DR(L)-CAGGS-eGFP-pA-IR-DR(R) plasmid was replaced with the native rat Elongation Factor-1 alpha promoter (rEF1a) from pDRIVE-rEF1 $\alpha$  (InvivoGen). Fragment PstI-NcoI containing rEF1a was blunted and inserted into the NotI-MluI blunted backbone. Screening for “sense” orientation was by EcoRI digestion. Functional elements of sleeping beauty construct were confirmed by sequencing. The SB100x transposase was prepared following protocol by Mates et al (Mates, 2011).

*Synchronization of oestrus cycle in rat recipients:* The foster females aged 8 to 12 weeks were injected intraperitoneally (i.p) with 40ug of luteinizing hormone-releasing hormone (LH-RH) agonist 4 days prior to mating. On the day of mating, the recipients were individually placed with vasectomised males. The following day, the females were de-mated and examined for the presence of copulation plug. The females presenting a plug were used as embryo recipient for embryo transfer surgery on the same day.

*Embryo preparation and microinjection:* 6 SHR and 9 WKY females aged 4 to 6 weeks old were used as embryos donors. 2 days prior to mating, females were

injected i.p with 30 IU of pregnant mares serum gonadotropin (PMSG). On the day of mating, the females were injected i.p with 40 IU of human pregnancy urine chorionic gonadotropin (HCG) and placed individually with SHR or WKY males. The following day, the females were separated, culled and the oviducts removed. The oviducts were then placed in M2 media (Sigma) and the ampulla torn to release single cell embryos. Hyaluronidase was then added to the media to remove the cumulus cells. The embryos were collected and cleaned using a mouth pipette. The fertilized embryos were transferred from M2 to KSOMaa media (Zenith biotech) and left in the incubator at 37°C, 5% CO<sub>2</sub> until they were used for pro nuclear injections and embryo transfer (20-40 embryos per female) using standard protocols (Filipiak and Saunders, 2006; Geurts et al., 2009). Briefly, pronuclear injection of mixed donor plasmid (0.4 ng/ul) and mRNA SB100X transposase (5 ng/ul) was performed in WKY and SHR rat one cell embryos. Injected embryos were transferred to the oviduct of day-0.5 pseudopregnant rats and then replaced into Sprague Dawley rat recipients by embryo transfer surgery.

### **Screening of GFP positive pups.**

Selected F0 founders were crossed with wild types and F1 were screened by PCR and GFP-positive animals were further screened for GFP expression in blood to choose high expressers for breeding. Genomic DNA was extracted from ear clips by overnight proteinase K digestion at 56°C and precipitated next day with isopropanol. PCR with two primer pairs was performed to detect 343bp Actin fragment as control and 180bp fragment as transgene positive.

DNA\_rat\_bActin\_F: 5'-TGTGTTGTCCCTGTATGCCTCT-3'

DNA\_rat\_bActin\_R: 5'- ATTGCCGATAGTGATGACCTGA-3'



GFP\_F: 5'-GGCACCTACCCCAGCGGCTA-3'

GFP\_R: 5'-CCGGTGCCCACCACCTTGAA-3'

PCR cycle conditions were: 95°C for 2 min, 30 cycles of 95°C for 30 sec, 60°C for 30 sec, and 72°C for 30 sec with a final 5 min elongation at 72°C. Tail blood was collected into lithium-heparin microvette tubes and GFP blood expression was assessed using an AccuriC6 (BD) flow cytometer (see 'detection of GFP expression' below). Whole animal GFP expression was confirmed under an excitation light (489nm) (Fig. 1B).

### **Integration sites identification and locus confirmation**

Ligation-mediated PCR (LMPCR) (Ivics et al., 2014) was used to locate the transgene integration site in the host genome. We designed primers to combine with Tba1 5'-CTTGTGTCATGCACAAAGTAGATGTCC-3' from the LMPCR protocol to verify individual transgene integration by locus-specific PCR:

Ch1\_is\_F: 5'-GACACATCCCTTGCTGTGGA-3'

Ch1\_is\_R: 5'-GCTATAACAAAGGGGACAGGCT-3';

Ch5\_is\_F: 5'-CTCCAGCTTGCTTCTTGGA-3'

Ch5\_is\_R: 5'-ACTGCACCCCAGAAAAAGCA-3';

Ch8\_is\_F: 5'-GAAACACTGCACGTGGTGAC-3'

Ch8\_is\_R: 5'-TCTCTTCCCACAGCCTTTCC-3'

### **Detection of GFP expression**

*Embryos:* E4.5 embryos were collected and carefully placed in glass bottom petri dishes for confocal imaging, using Leica Sp5 microscope. For some embryos, E4.5 were harvested and cultured overnight (12hrs) in M2 media before imaging.

*Organs:* Brain, eye, salivary gland, thymus, heart, lung, liver, kidney, adrenal gland gut, pancreas, spleen, and quadriceps were dissected, washed, fixed (4% PFA 30mins) and mounted on open glass slides for imaging using a Leica MZ16F fluorescence stereoscope and Leica camera DFC420C. For some organs, longitudinal dissection was used to show GFP intensity of inner organ structures. In addition, kidney, liver, spleen and thymus gross dissections were embedded in OCT and 2µm frozen sections mounted onto slides using hard set mounting media with DAPI (vectashield). Sections were imaged using upright fluorescent microscope (Olympus) for GFP and DAPI.

*Blood:* Blood was collected into lithium-heparin microvette tubes from tail bleeds. Red blood cells were lysed (155mM NH<sub>4</sub>Cl, 12mM NaHCO<sub>3</sub>, 100mM EDTA) and leukocytes washed in PBS before permeabilisation (fix-Perm kit, Invitrogen) and incubation with antibodies against rat Gran-PE (HIS48), MHC-II-perCP (HIS19) and CD68-alexa700 (ED1) (all 1:50; Biologend). Cells were analysed by flow cytometry using an AccuriC6 (BD) with appropriate colour compensation and gates set to unstained cells.

### **Culture of bone marrow-derived macrophages**

Rat bone marrow-derived macrophages were isolated from femurs and differentiated using RPMI 1640 supplemented with 10% Fetal Bovine Serum (Hyclone) and 5 ng/ml Rat M-CSF (Peprotech) as previously described (D'Souza et al., 2013). Cells were cultured in 12 well plates for 5 or 10 days and GFP fluorescence images were taken using confocal microscopy.

## Intravital microscopy

To examine utility of GFP Tg rats for translational work, we tested the real-time imaging of kidney leukocyte-endothelial interactions using intravital microscopy in WKY-GFP bone marrow chimeras. WKY-GFP bone marrow was harvested and injected into WKY wild type recipient to create bone marrow chimeras as previously described (Smith et al., 2007). Briefly, femurs were harvested from WKY-GFP rats and bone marrow cells collected under sterile conditions. Recipient WKY rats were irradiated (8Gy) and bone marrow cells (approx.  $8 \times 10^6$ ) injected intravenously (i.v). After 8 weeks, reconstitution was confirmed (data not shown) and kidney imaged under anaesthesia, after exposure of superficial kidney cortex from surrounding tissue by careful blunt dissection. Images were taken using SP5 confocal microscope with i.v infusion of 70kDa- dextran tetramethylrhodamine (Invitrogen).

## Results

### Microinjection results

Seventy-five percent of newborn pups after microinjections were GFP positive by direct inspection under UV light and by PCR. PCR assay results corresponded exactly to GFP expression by direct inspection in both WKY-GFP and SHR-GFP rat lines (Table 1).

Two positive F<sub>0</sub> founders from each strain were crossed to wild type to confirm transgene germline transmission. All F<sub>0</sub> founders transmitted the transgene. Only one high GFP expresser F<sub>0</sub> per strain was used to derive each transgenic (Tg) rat line. Both lines showed normal growth, were able to reproduce, germline transmit the transgene and after more than five generations, GFP expression was maintained without any sign of transgene silencing.

### Examination of the GFP Integration site

Ligation-mediated PCR (LMPCR) protocol (Ivics et al., 2014) was used to locate the transgene integration site in the host genome. GFP transgene insertion sites were located using Ensembl genome browser, Rat (Rnor\_6.0).

Two integration sites were identified in the WKY-GFP founder of the Tg line on Chromosome 8:28170658 and Chromosome 1:276465837, both located in intronic gene areas, *Jam3* (ENSRNOG00000009149) intron 1 and *Vti1a* (ENSRNOG00000042786) intron 6 respectively (Fig. 1C). The *Jam3* intron 1 is 51319bp long, and the transgene resides at 37.8kb from exon 1 and 13.5kb from exon 2. In *Vti1a* intron 6, 102565 bp long, the transgene is located at 81.4kb from exon 5 and 21.1kb from exon 6.

One single location was identified in the SHR-GFP founder of the Tg line on Chromosome 5:108698150, an intergenic area where the closest gene, *Zfp353* (ENSRNOG00000050804) is located at 47.9kb downstream (Fig. 1C).

### **GFP expression in embryos, tissues and blood**

To examine the level of GFP expression in tissues from Tg WKY and SHR rats; blood, cells and organs were processed for fluorescence microscopy. Figure 2 shows representative examples of GFP expression in E4.5 early blastocyst embryos from homozygote WKY-GFP female crossed with wild type WKY male. All embryos were GFP bright which sustained for at least 12hrs in culture (Fig. 2, lower panel). Similar expression was noted in WKY-GFP homozygotes and SHR-GFP rats (data not shown).

We next isolated brain, eye, salivary glands, thymus, heart, lung, liver, kidney, adrenal glands, gut, pancreas, spleen, and quadriceps from wildtype (SHR), WKY-GFP and SHR-GFP rats. All organs displayed bright GFP expression with some differences in GFP intensity between and within organs (Fig. 3 and Fig. S1). In general GFP intensity was moderately less in SHR rats, although both strains expressed GFP in all organs and tissues investigated (Fig. 3). Interestingly, small variations in GFP expression were observed in heart, kidney, thymus and brain tissue, localised to specific areas within the organs (Fig. 3). This may reflect differences in *rEF1a* expression in different cell types.

GFP intensity was also maintained after tissue histological processing. Kidney, liver, spleen and thymus were processed for frozen sectioning and stained for GFP and

DAPI expression. All tissues were GFP bright, with some differences in GFP expression across the tissue (Fig. S2).

We next analysed GFP expression in blood cell populations using flow cytometry. All blood leukocyte populations, based on either SSC<sup>high</sup> or SSC<sup>low</sup> cells expressed similar amounts of GFP intensity in both WKY-GFP and SHR-GFP rats (Fig. 4A). However, after gating for either granulocytes (Gran), MHC-II or CD68 there was noticeable differences in GFP expression on some subpopulations in SHR-GFP rats (Fig. 4B). Similar data was noted for WKY-GFP rats (data not shown). CD68<sup>neg</sup>Gran<sup>pos</sup>MHC-II<sup>high</sup> cells, which are most likely lymphocyte and some granulocyte populations, expressed the highest level of GFP (Fig. 4B), whereas CD68<sup>pos</sup> (monocytes) are GFP intermediate and GFP<sup>low</sup> expressing cells are within Gran<sup>pos</sup> and MHC-II<sup>high</sup> populations (Fig. 4B). The differences may reflect *rEF1a* expression in different circulating subpopulations.

### **Bone marrow derived macrophage culture in-vitro**

To examine if GFP could be sustained in primary cell culture passage, which would be useful for downstream experimental applications, we examined GFP expression in bone marrow-derived macrophages (BMDM). BMDMs cultured over 5 and 10 days sustained high GFP expression, as detected by fluorescence microscopy (Fig. 5A). There was no difference in WKY-GFP or SHR-GFP BMDM GFP expression and similar amounts of GFP were expressed between 5 and 10 days (Fig. 5A). This indicates that within proliferating macrophage cultures, *rEF1a* activity and consequently GFP intensity is maintained in daughter cells.

### **In-vivo bone marrow chimeras and intravital microscopy**

To further examine the utility of Tg rat GFP lines in potential disease models and *in vivo* applications, we examined GFP intensity in WKY-GFP bone marrow chimera experiments using intravital microscopy. WKY-GFP BMT was performed in WKY recipients and the kidney cortex exposed for confocal imaging. Figure 5B shows that GFP bright cells can be seen located at the endothelial interface.

## Discussion

The potential of rat models for understanding basic biology and human health and disease (Aitman et al., 2008) has increased owing to availability of new gene targeting tools applicable to the rat (Atanur et al., 2013; Jacob et al., 2010) and communal efforts to increase and refine rat genomic resources. Important aims of creating Tg rat models, or any Tg animal model, are to achieve high transgenesis efficiency with strong and permanent expression of the gene of interest through generations, with high efficiency to minimise use of resources and animal numbers. The efficiency of transgenesis has increased in recent years due to development and refinement of new systems, from low efficiency of classical transgenesis by microinjection of naked DNA (Charreau et al., 1996; Filipiak and Saunders, 2006) to higher efficiency with retroviral transgenesis and the latest lentivirus vectors (Michalkiewicz et al., 2007). The Sleeping Beauty transposon system has advantages for germline transgenesis as a simpler and safer delivery system, matching or exceeding other approaches. This approach with a single copy of a permanent insertion in the genome leads to preferential intergenic integrations, and is less prone to silencing (Ivics et al., 2014; Katter et al., 2013; Mates, 2011; Mates et al., 2009; Park, 2007).

We have created two new transgenic lines, on the WKY and SHR genetic backgrounds, that display and take advantage of many of these characteristics. In our experiments, 75% of new F0 born pups were transgene positive (Table 1), one of the highest transgenesis efficiencies reported. Such efficiency, in our case, seems independent of the SHR or WKY genetic rat background. In our Tg models, GFP



expression appeared to be independent of integration site and genomic background and dependant of promoter endogenous/native activity. Further, expression was transmitted unchanged through more than five generations of breeding and through 5-10 days of in vitro macrophage culture, demonstrating the stability of expression from the transgene constructs integrated into the germline genome. Finally, the high level of GFP expression was shown to have created a suitable platform for cell fate tracking through intravital microscopy and confocal imaging.

Avoiding the production of mosaic founders is an important way to minimise cost, and increasing effective functional efficiency. Sleeping beauty is a plasmid/mRNA based method where the transposase mRNA does not need embryonic genome activation. In rats this begins at the 4-cell stage and only needs translation of the transposase mRNA in the early embryo stages. This system largely avoids the creation of mosaic founders (Mates, 2011). This is superior to lentivirus transgenesis where reverse transcription is necessary and where viral infection and integration can continue with embryonic development (Park, 2007). The transmission of the transgene over 5 generations in our lines excludes the possibility of mosaicism in the selected founders of our established WKY and SHR lines.

The Sleeping Beauty transposon system randomly integrates the gene of interest in the genome with a bias to intergenic integration (Ammar et al., 2012; Grabundzija et al., 2010; Katter et al., 2013; Moldt et al., 2011). We show in our new GFP Tg rat models, by locating and verifying the integration locus sites, that there was no functional gene disruption due to exon insertion. We confirmed individual transgene integration by locus-specific PCR, making possible the isolation of each integration,

by selecting breeding and establishing unique locus integration per rat line. In the SHR inbred background the insertion position was at Chromosome 5:108698150, an intergenic locus 47.9kb upstream of gene *Zfp353*. In such a location, using the rat genome information available (Rnor\_6.0), we cannot foresee any biological impairment. In the WKY inbred background the two located integration sites were intronic, one on Chromosome 1:276465837, intron 6 (102,565bp long) of gene *Vti1a* (Vesicle transport through interaction with t-SNAREs 1A) and the other on Chromosome 8:28170658, intron 1 (51319bp) of gene *Jam3* (Junctional adhesion molecule 3). Regulatory splicing elements can be found in the intron up to 300bp from the exon-intron border (Barash et al., 2010). Our intronic integrations are far away from these areas, being ~13.5kb from the closest exon-intron border. Therefore effects on the splicing machinery would not be foreseen although interference due to Tg insertion in introns, which regulate gene expression by noncoding RNA molecules, intron-mediated enhancement or by other intronic regulatory functions, cannot be completely excluded.

To ensure strong and permanent expression, many factors are necessary, including a strong promoter and fortuitous integration in the genome to avoid transgene silencing (Kioussis and Festenstein, 1997). The selection of promoter was fundamental to accomplish this goal. We decided to move away from the most used, the chimeric exogenous CAG promoter because it contains the cytomegalovirus enhancer sequence and due to its viral nature could trigger silencing mechanisms affecting GFP expression. Moreover, although CAG promoters are ubiquitous, it has been reported there are different expression patterns within different tissues and within different transgenic models, from high expression to low or no expression

(Hakamata et al., 2001; Inoue et al., 2005; Katter et al., 2013; Michalkiewicz et al., 2007; Murakami and Kobayashi, 2012; Popova et al., 2008; Takeuchi et al., 2003). We therefore opted for an endogenous promoter to maximise the likelihood of permanent expression and escape from gene silencing. Most promoters that have been used have some disadvantages. For example rat transgenic models using Ubiquitin C (van den Brandt et al., 2004) , phosphoglycerate kinase 1 (PGK1) (Remy et al., 2010) and Rosa26 (Montanari et al., 2014) promoters report chimeric expression. Potential promoter candidates were compared using the on-line BioGPS application (<http://biogps.org>) where the tissue-specific pattern of mRNA expression of their endogenous driven protein is quantified. We hypothesized that the expression of GFP will mimic our gene of interest, at least to an extent, to the reported expression. Eukaryotic Elongation factor 1 alpha 1 (EEF1A1) or rat Elongation factor 1 alpha (rEF1a) has one of the most wide-ranging tissues expression patterns, with the highest median (M= 10426.3), followed by Ubiquitin C (M=4620.6) and PGK1 (M=4.5). One possible weakness of rEF1a is the low expression in heart and skeletal muscle tissues. However, given the near ubiquitous expression, we selected the rEF1a promoter for our Tg models.

In our Tg models, GFP expression seems to be independent of integration site and genomic background and dependant of promoter endogenous/native activity. We visually confirmed that GFP expression follows the pattern of *rEF1a* mRNA expression, with a markedly lower expression in the heart, in both SHR and WKY rats, implying possibly that the rEF1a promoter is the main determinant in these strains of the level of tissue GFP expression. It would be interesting to perform further studies to quantify *gfp* mRNA versus *rEF1a* mRNA in tissues to assess

transcriptional correlation. If true, these models could be used as *rEF1a* expression reporter, and also as a *rEF1a* canonical function assay to deliver aa-tRNA to ribosome in mRNA translation. It could well be a reporter for cellular translation levels. The difference in GFP intensity between the SHR and WKY Tg models could be due to transgene copy, SHR with a single copy shows relatively lower expression of GFP than WKY with two integrations. Finally, heterogenous GFP expression within a tissue, such as the brain, may reflect differing chromatin state of the integration site. One hypothesis could be that, since the *rEF1a* promoter is essential in cellular protein translation, integration could make the chromatin more accessible in particular cell types if these loci are normally closed when translation occurs. Further work is needed to confirm whether these models reflect *rEF1a* expression in specific tissues and/or chromatin state after integration.

In summary, we have created two novel Tg models expressing ubiquitous green fluorescent protein in WKY and SHR inbred rat lines to support in vivo and ex vivo studies in cell tracking, tissue and organ transplantation for further elucidation of the complex disease traits of human crescentic glomerulonephritis (CRGN) and metabolic syndrome respectively. Our preliminary study using real time imaging of kidney leukocyte-endothelial interactions in WKY-GFP bone marrow chimeras confirms the utility of GFP transgenic rats for fluorescent imaging. Both novel Tg lines have considerable value for future translational research in the scientific community.

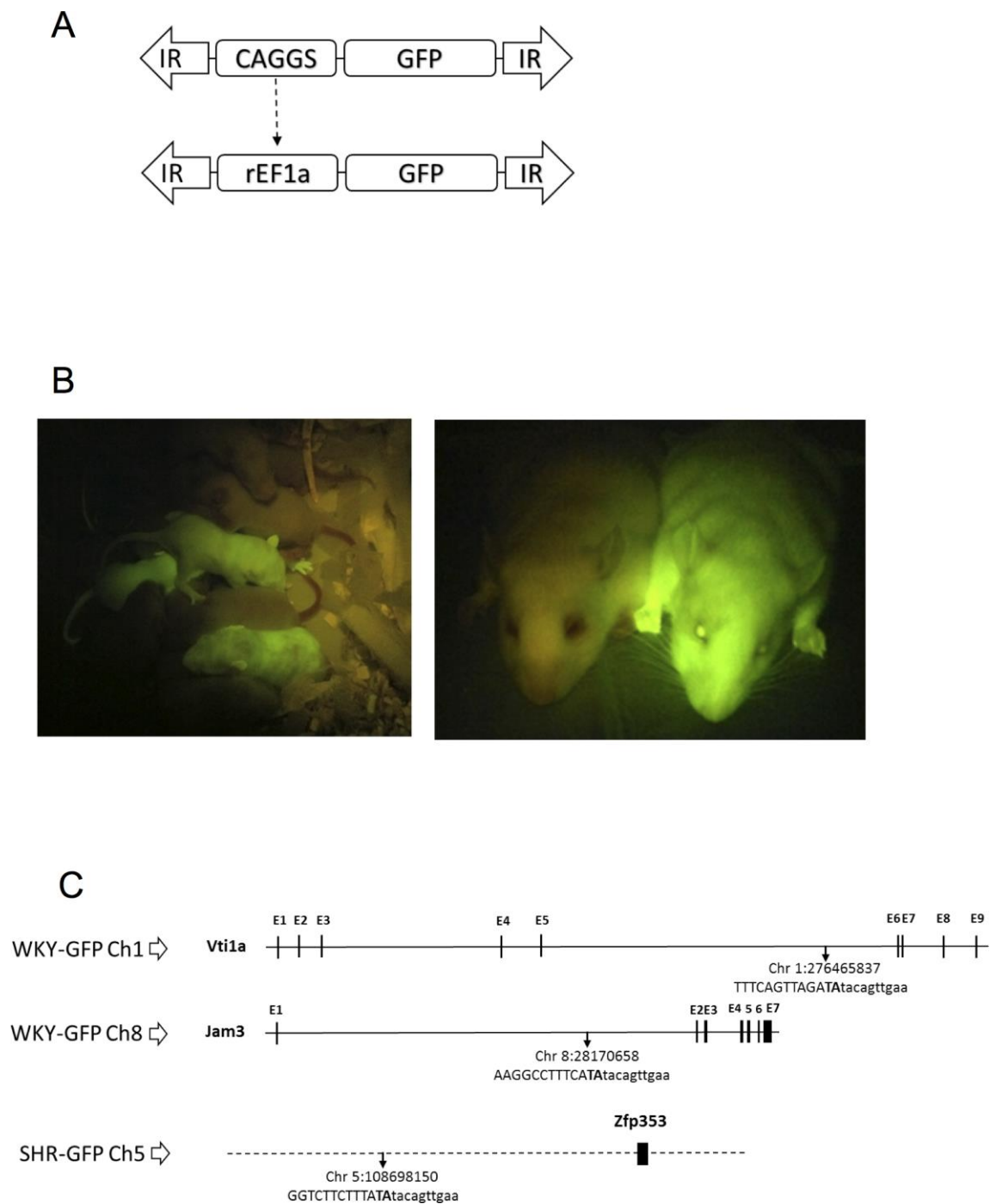
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## Tables

<b>Table 1</b> Overall transgenesis efficiency by SB transposon system				
	No of zygotes surviving microinjection and transferred to recipients. (Survival rate, %)	No. of pregnant recipients (%)	Efficiency of transposon integration (%)	
			Per new born	Per injected zygotes
<b>WKY-GFP</b>	22/85 (25.8)	1/1 (100)	3/4 (75)	3/22 (13.6)
<b>SHR-GFP</b>	96/270 (35.5)	3/3 (100)	6/8 (75)	6/96 (6.25)

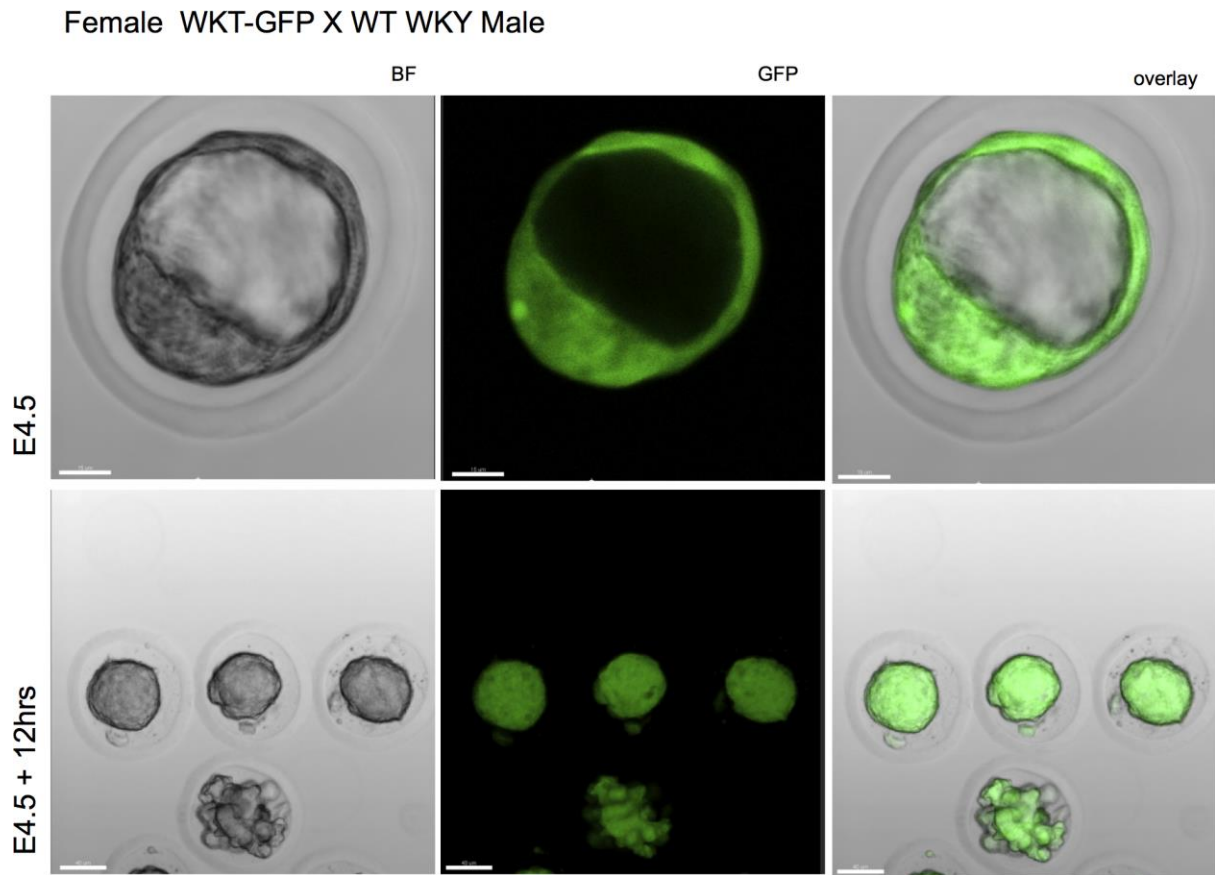
Figures



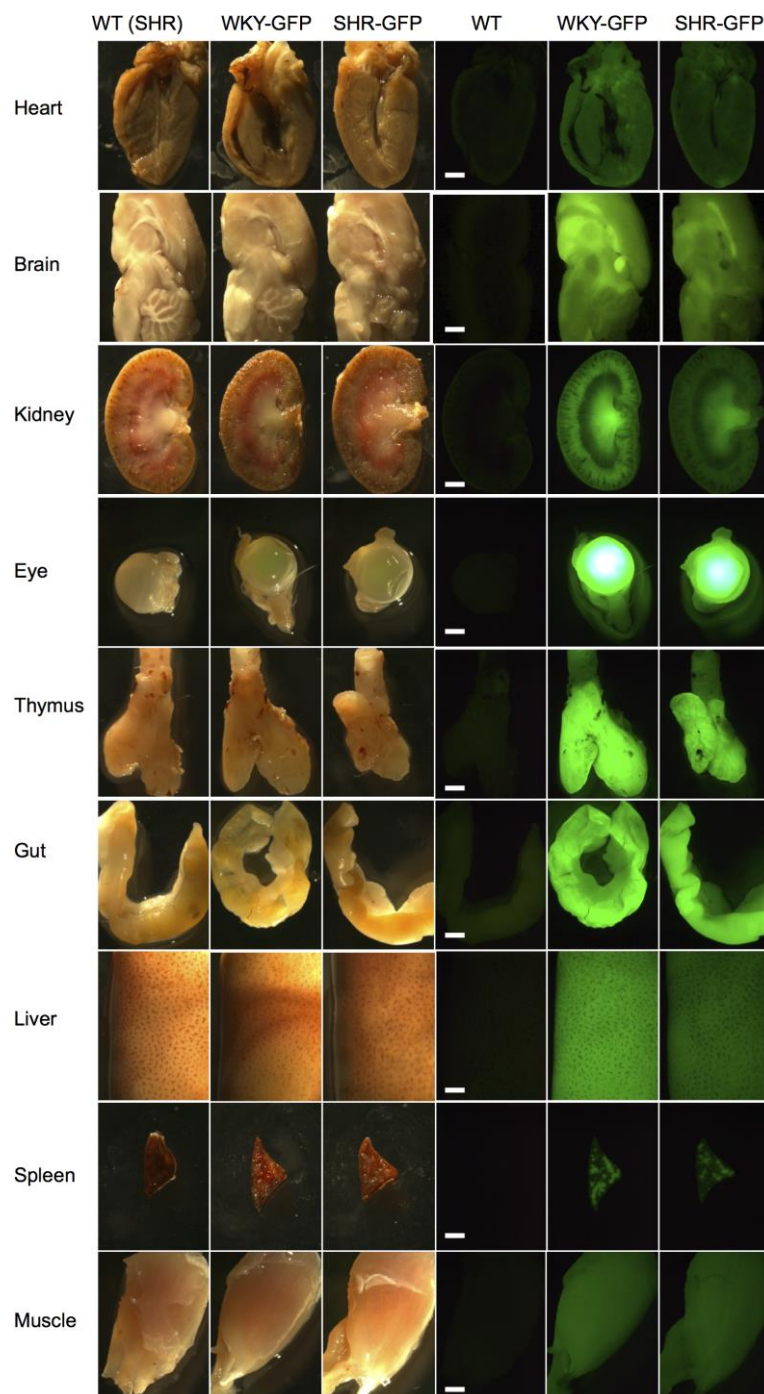
**Figure 1. GFP transgenic rat design.** (A) Schematic plasmid representation. rat Elongation Factor 1 alpha promoter (rEF1a) replaces CAG promoter (CAGGS). IR =

Inverted repeats; GFP = Green Fluorescent Protein cDNA (GFP). **(B)** Photograph of WKY-GFP pups (left) and adult (right) rats under excitation light 489nm, showing wild type and GFP Tg animals. **(C)** Schematic genome locus showing TA integration sites location of T for SHR-GFP in chromosome 5, and WKY-GFP in chromosome 1 and 8. Genomic sequence (left junction) in capitals and transgene in lowercase, dotted horizontal line refers to intergenic, continuous black line to intronic, and vertical blocks to exonic sequences.

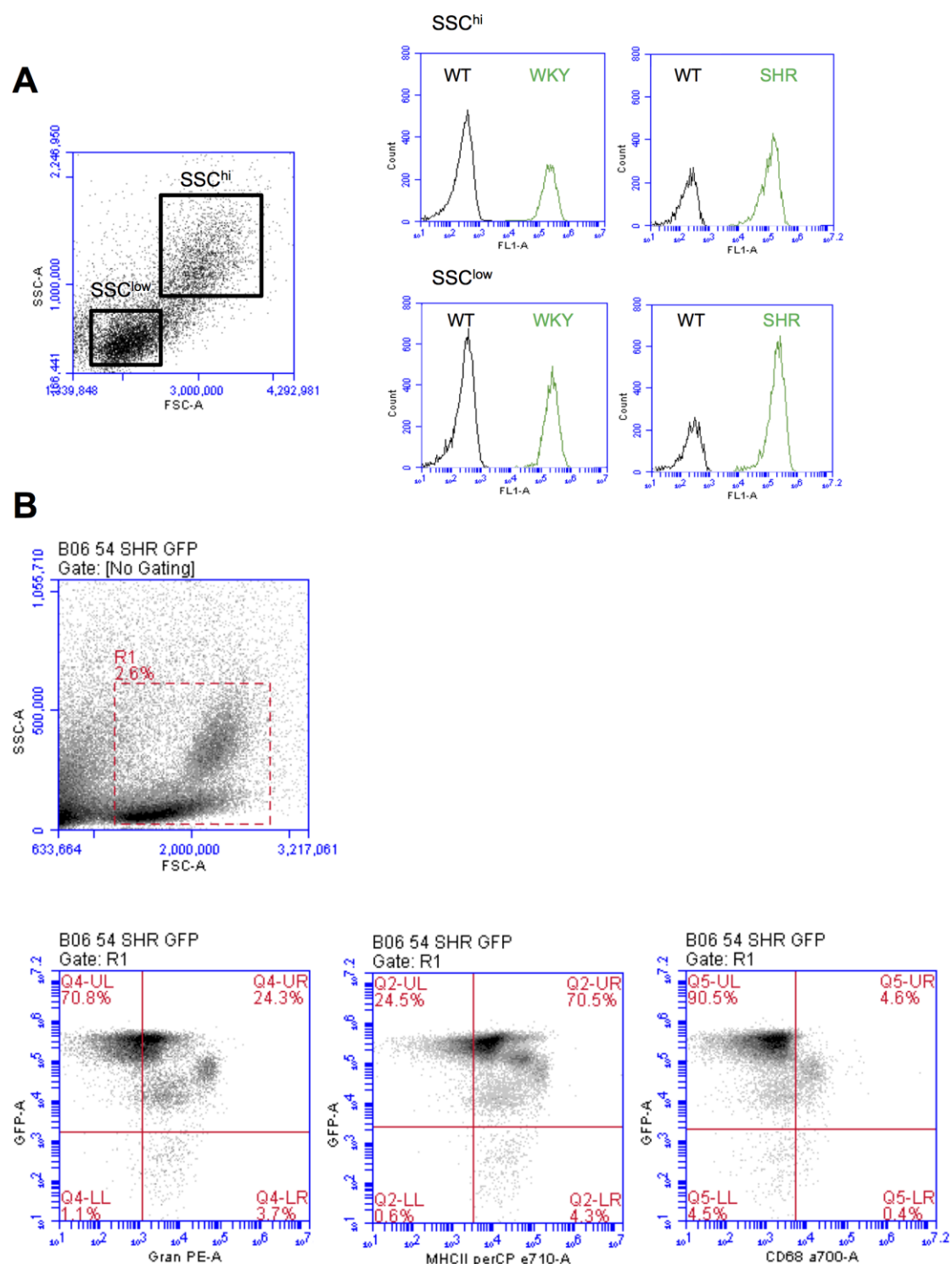




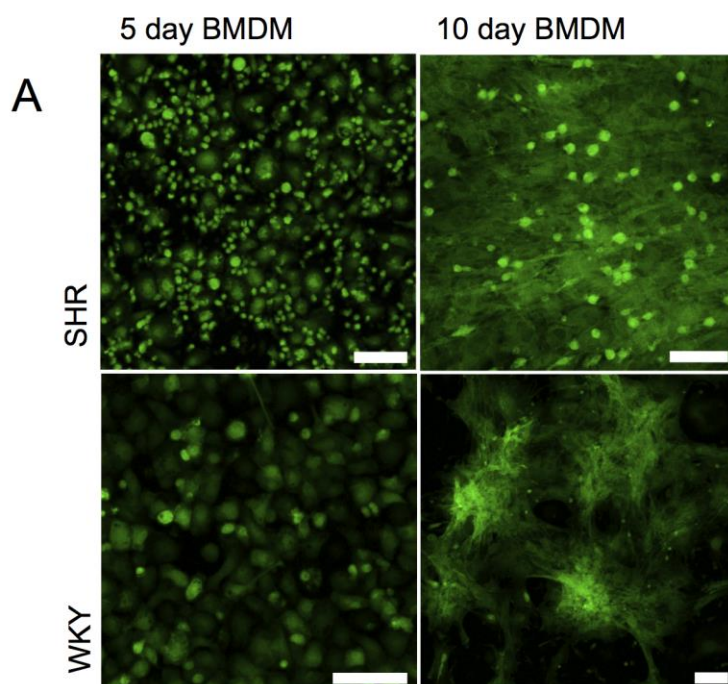
**Figure 2. GFP expression in embryos.** Female WKT-GFP were crossed with wildtype WKY male rats and one-cell embryos removed and imaged under confocal microscope. Representation of 3 experiments from one-cell embryos at E4.5 and E4.5 plus 12hrs. Scale bar = 15 $\mu$ m top panel and 40 $\mu$ m bottom panel.



**Figure 3. GFP expression in organs and tissues.** Wild type (from SHR strain), WKY-GFP and SHR-GFP rats were examined for gross GFP expression in dissected heart, brain, kidney, eyes, thymus, gut, liver, spleen and muscle tissues. Whole organ extracts were mounted for stereo- fluorescent imaging. Scale bar = 2mm.



with or without fluorescent labelled antibodies using flow cytometry. (A). Histograms of GFP expression in SSC<sup>hi</sup> and SSC<sup>low</sup> populations. (B). Dot plots of GFP expression versus Gran, MHC-II or CD68 positive populations, gated from R1. Representative of at least n=4 rats.



**B**

Snapshot: Intravital microscopy WKY<sup>GFP</sup> BMT  
Kidney cortex



**Figure 5. GFP expression in BMDM and intravital microscopy.** (A). Bone marrow derived macrophages (BMDM) were harvested and cultured for 5 or 10 days and intensity of GFP examined in WKY-GFP and SHR-GFP rats. Scale bar = 75µm. (B).

WKY rats underwent irradiation and BM transplant from WKY-GFP donor. After successful chimerisation, kidney cortex was imaged after injection of 70kDa fluorescent dextran under anaesthesia. Snapshot is shown of GFP positive cells interacting with endothelial surfaces within the kidney (arrows). Representative of at least n=4 rats.



## References

- Aitman, T.J., Critser, J.K., Cuppen, E., Dominiczak, A., Fernandez-Suarez, X.M., Flint, J., Gauguier, D., Geurts, A.M., Gould, M., Harris, P.C., *et al.* (2008). Progress and prospects in rat genetics: a community view. *Nat Genet* 40, 516-522.
- Aitman, T.J., Dong, R., Vyse, T.J., Norsworthy, P.J., Johnson, M.D., Smith, J., Mangion, J., Robertson-Lowe, C., Marshall, A.J., Petretto, E., *et al.* (2006). Copy number polymorphism in *Fcgr3* predisposes to glomerulonephritis in rats and humans. *Nature* 439, 851-855.
- Aitman, T.J., Glazier, A.M., Wallace, C.A., Cooper, L.D., Norsworthy, P.J., Wahid, F.N., Al-Majali, K.M., Trembling, P.M., Mann, C.J., Shoulders, C.C., *et al.* (1999). Identification of *Cd36* (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 21, 76-83.
- Aitman, T.J., Gotoda, T., Evans, A.L., Imrie, H., Heath, K.E., Trembling, P.M., Truman, H., Wallace, C.A., Rahman, A., Dore, C., *et al.* (1997). Quantitative trait loci for cellular defects in glucose and fatty acid metabolism in hypertensive rats. *Nat Genet* 16, 197-201.
- Ammar, I., Gogol-Doring, A., Miskey, C., Chen, W., Cathomen, T., Izsvak, Z., and Ivics, Z. (2012). Retargeting transposon insertions by the adeno-associated virus Rep protein. *Nucleic acids research* 40, 6693-6712.
- Atanur, S.S., Birol, I., Guryev, V., Hirst, M., Hummel, O., Morrissey, C., Behmoaras, J., Fernandez-Suarez, X.M., Johnson, M.D., McLaren, W.M., *et al.* (2010). The genome sequence of the spontaneously hypertensive rat: Analysis and functional significance. *Genome Res* 20, 791-803.
- Atanur, S.S., Diaz, A.G., Maratou, K., Sarkis, A., Rotival, M., Game, L., Tschannen, M.R., Kaisaki, P.J., Otto, G.W., Ma, M.C., *et al.* (2013). Genome sequencing reveals loci under artificial selection that underlie disease phenotypes in the laboratory rat. *Cell* 154, 691-703.
- Barash, Y., Calarco, J.A., Gao, W., Pan, Q., Wang, X., Shai, O., Blencowe, B.J., and Frey, B.J. (2010). Deciphering the splicing code. *Nature* 465, 53-59.
- Behmoaras, J., Bhangal, G., Smith, J., McDonald, K., Mutch, B., Lai, P.C., Domin, J., Game, L., Salama, A., Foxwell, B.M., *et al.* (2008). *Jund* is a determinant of macrophage activation and is associated with glomerulonephritis susceptibility. *Nature genetics* 40, 553-559.
- Behmoaras, J., Diaz, A.G., Venda, L., Ko, J.H., Srivastava, P., Montoya, A., Faull, P., Webster, Z., Moyon, B., Pusey, C.D., *et al.* (2015). Macrophage epoxygenase determines a profibrotic transcriptome signature. *J Immunol* 194, 4705-4716.
- Bishop, J.O., and Smith, P. (1989). Mechanism of chromosomal integration of microinjected DNA. *Mol Biol Med* 6, 283-298.
- Charreau, B., Tesson, L., Soullillou, J.P., Pourcel, C., and Anegon, I. (1996). Transgenesis in rats: technical aspects and models. *Transgenic Res* 5, 223-234.

D'Souza, Z., McAdoo, S.P., Smith, J., Pusey, C.D., Cook, H.T., Behmoaras, J., and Aitman, T.J. (2013). Experimental crescentic glomerulonephritis: a new bicongenic rat model. *Disease models & mechanisms* 6, 1477-1486.

Ellis, J. (2005). Silencing and variegation of gammaretrovirus and lentivirus vectors. *Hum Gene Ther* 16, 1241-1246.

Filipiak, W.E., and Saunders, T.L. (2006). Advances in transgenic rat production. *Transgenic Res* 15, 673-686.

Garrick, D., Fiering, S., Martin, D.I., and Whitelaw, E. (1998). Repeat-induced gene silencing in mammals. *Nat Genet* 18, 56-59.

Geurts, A.M., Cost, G.J., Freyvert, Y., Zeitler, B., Miller, J.C., Choi, V.M., Jenkins, S.S., Wood, A., Cui, X., Meng, X., *et al.* (2009). Knockout rats via embryo microinjection of zinc-finger nucleases. *Science* 325, 433.

Gill, D.R., Smyth, S.E., Goddard, C.A., Pringle, I.A., Higgins, C.F., Colledge, W.H., and Hyde, S.C. (2001). Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. *Gene therapy* 8, 1539-1546.

Grabundzija, I., Irgang, M., Mates, L., Belay, E., Matrai, J., Gogol-Doring, A., Kawakami, K., Chen, W., Ruiz, P., Chuah, M.K., *et al.* (2010). Comparative analysis of transposable element vector systems in human cells. *Mol Ther* 18, 1200-1209.

Hakamata, Y., Tahara, K., Uchida, H., Sakuma, Y., Nakamura, M., Kume, A., Murakami, T., Takahashi, M., Takahashi, R., Hirabayashi, M., *et al.* (2001). Green fluorescent protein-transgenic rat: a tool for organ transplantation research. *Biochem Biophys Res Commun* 286, 779-785.

Hofmann, A., Kessler, B., Ewerling, S., Kabermann, A., Brem, G., Wolf, E., and Pfeifer, A. (2006). Epigenetic regulation of lentiviral transgene vectors in a large animal model. *Mol Ther* 13, 59-66.

Hubner, N., Wallace, C.A., Zimdahl, H., Petretto, E., Schulz, H., Maciver, F., Mueller, M., Hummel, O., Monti, J., Zidek, V., *et al.* (2005). Integrated transcriptional profiling and linkage analysis for identification of genes underlying disease. *Nat Genet* 37, 243-253.

Iannaccone, P.M., and Jacob, H.J. (2009). Rats! *Disease models & mechanisms* 2, 206-210.

Inoue, H., Ohsawa, I., Murakami, T., Kimura, A., Hakamata, Y., Sato, Y., Kaneko, T., Takahashi, M., Okada, T., Ozawa, K., *et al.* (2005). Development of new inbred transgenic strains of rats with LacZ or GFP. *Biochem Biophys Res Commun* 329, 288-295.

Ivics, Z., Li, M.A., Mates, L., Boeke, J.D., Nagy, A., Bradley, A., and Izsvak, Z. (2009). Transposon-mediated genome manipulation in vertebrates. *Nat Methods* 6, 415-422.



Ivics, Z., Mates, L., Yau, T.Y., Landa, V., Zidek, V., Bashir, S., Hoffmann, O.I., Hiripi, L., Garrels, W., Kues, W.A., *et al.* (2014). Germline transgenesis in rodents by pronuclear microinjection of Sleeping Beauty transposons. *Nature protocols* 9, 773-793.

Jacob, H.J., Lazar, J., Dwinell, M.R., Moreno, C., and Geurts, A.M. (2010). Gene targeting in the rat: advances and opportunities. *Trends Genet* 26, 510-518.

Kanno, K., Okumura, F., Toriumi, W., Ishiyama, N., Nishiyama, S., and Naito, K. (1998). Nephrotoxic serum-induced nephritis in Wistar-Kyoto rats: a model to evaluate antinephritic agents. *Japanese journal of pharmacology* 77, 129-135.

Katter, K., Geurts, A.M., Hoffmann, O., Mates, L., Landa, V., Hiripi, L., Moreno, C., Lazar, J., Bashir, S., Zidek, V., *et al.* (2013). Transposon-mediated transgenesis, transgenic rescue, and tissue-specific gene expression in rodents and rabbits. *FASEB J* 27, 930-941.

Kim, D.W., Uetsuki, T., Kaziro, Y., Yamaguchi, N., and Sugano, S. (1990). Use of the human elongation factor 1 alpha promoter as a versatile and efficient expression system. *Gene* 91, 217-223.

Kioussis, D., and Festenstein, R. (1997). Locus control regions: overcoming heterochromatin-induced gene inactivation in mammals. *Current opinion in genetics & development* 7, 614-619.

Kitada, K., Ishishita, S., Tosaka, K., Takahashi, R., Ueda, M., Keng, V.W., Horie, K., and Takeda, J. (2007). Transposon-tagged mutagenesis in the rat. *Nat Methods* 4, 131-133.

Kurtz, T.W., and Morris, R.C., Jr. (1987). Biological variability in Wistar-Kyoto rats. Implications for research with the spontaneously hypertensive rat. *Hypertension* 10, 127-131.

Lepretre, F., Vasseur, F., Vaxillaire, M., Scherer, P.E., Ali, S., Linton, K., Aitman, T., and Froguel, P. (2004). A CD36 nonsense mutation associated with insulin resistance and familial type 2 diabetes. *Hum Mutat* 24, 104.

Lois, C., Hong, E.J., Pease, S., Brown, E.J., and Baltimore, D. (2002). Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors. *Science* 295, 868-872.

Mates, L. (2011). Rodent transgenesis mediated by a novel hyperactive Sleeping Beauty transposon system. *Methods Mol Biol* 738, 87-99.

Mates, L., Chuah, M.K., Belay, E., Jerchow, B., Manoj, N., Acosta-Sanchez, A., Grzela, D.P., Schmitt, A., Becker, K., Matrai, J., *et al.* (2009). Molecular evolution of a novel hyperactive Sleeping Beauty transposase enables robust stable gene transfer in vertebrates. *Nat Genet* 41, 753-761.

Michalkiewicz, M., Michalkiewicz, T., Geurts, A.M., Roman, R.J., Slocum, G.R., Singer, O., Weihrauch, D., Greene, A.S., Kaldunski, M., Verma, I.M., *et al.* (2007).

Efficient transgenic rat production by a lentiviral vector. *Am J Physiol Heart Circ Physiol* 293, H881-894.

Mizushima, S., and Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic acids research* 18, 5322.

Moldt, B., Miskey, C., Staunstrup, N.H., Gogol-Doring, A., Bak, R.O., Sharma, N., Mates, L., Izsvak, Z., Chen, W., Ivics, Z., and Mikkelsen, J.G. (2011). Comparative genomic integration profiling of Sleeping Beauty transposons mobilized with high efficacy from integrase-defective lentiviral vectors in primary human cells. *Mol Ther* 19, 1499-1510.

Montanari, S., Wang, X.H., Yannarelli, G., Dayan, V., Berger, T., Zocche, L., Kobayashi, E., Viswanathan, S., and Keating, A. (2014). Development and characterization of a new inbred transgenic rat strain expressing DsRed monomeric fluorescent protein. *Transgenic Res* 23, 779-793.

Mullins, J.J., Peters, J., and Ganten, D. (1990). Fulminant hypertension in transgenic rats harbouring the mouse Ren-2 gene. *Nature* 344, 541-544.

Murakami, T., and Kobayashi, E. (2012). GFP-transgenic animals for in vivo imaging: rats, rabbits, and pigs. *Methods Mol Biol* 872, 177-189.

Okamoto, K., and Aoki, K. (1963). Development of a strain of spontaneously hypertensive rats. *Japanese circulation journal* 27, 282-293.

Park, F. (2007). Lentiviral vectors: are they the future of animal transgenesis? *Physiol Genomics* 31, 159-173.

Popova, E., Rentzsch, B., Bader, M., and Krivokharchenko, A. (2008). Generation and characterization of a GFP transgenic rat line for embryological research. *Transgenic research* 17, 955-963.

Pravenec, M., Churchill, P.C., Churchill, M.C., Viklicky, O., Kazdova, L., Aitman, T.J., Petretto, E., Hubner, N., Wallace, C.A., Zimdahl, H., *et al.* (2008). Identification of renal Cd36 as a determinant of blood pressure and risk for hypertension. *Nat Genet* 40, 952-954.

Remy, S., Tesson, L., Usal, C., Menoret, S., Bonnamain, V., Nerriere-Daguin, V., Rossignol, J., Boyer, C., Nguyen, T.H., Naveilhan, P., *et al.* (2010). New lines of GFP transgenic rats relevant for regenerative medicine and gene therapy. *Transgenic Res* 19, 745-763.

Sado, Y., Okigaki, T., Takamiya, H., and Seno, S. (1984). Experimental autoimmune glomerulonephritis with pulmonary hemorrhage in rats. The dose-effect relationship of the nephritogenic antigen from bovine glomerular basement membrane. *Journal of clinical & laboratory immunology* 15, 199-204.

Sasikumar, A.N., Perez, W.B., and Kinzy, T.G. (2012). The many roles of the eukaryotic elongation factor 1 complex. *Wiley interdisciplinary reviews. RNA* 3, 543-555.

Serafini, M., Bonamino, M., Golay, J., and Introna, M. (2004). Elongation factor 1 (EF1alpha) promoter in a lentiviral backbone improves expression of the CD20 suicide gene in primary T lymphocytes allowing efficient rituximab-mediated lysis. *Haematologica* 89, 86-95.

Shen, B., Zhang, J., Wu, H., Wang, J., Ma, K., Li, Z., Zhang, X., Zhang, P., and Huang, X. (2013). Generation of gene-modified mice via Cas9/RNA-mediated gene targeting. *Cell Res* 23, 720-723.

Shimoyama, M., De Pons, J., Hayman, G.T., Laulederkind, S.J., Liu, W., Nigam, R., Petri, V., Smith, J.R., Tutaj, M., Wang, S.J., *et al.* (2015). The Rat Genome Database 2015: genomic, phenotypic and environmental variations and disease. *Nucleic acids research* 43, D743-750.

Smith, J., Lai, P.C., Behmoaras, J., Roufosse, C., Bhangal, G., McDaid, J.P., Aitman, T., Tam, F.W., Pusey, C.D., and Cook, H.T. (2007). Genes expressed by both mesangial cells and bone marrow-derived cells underlie genetic susceptibility to crescentic glomerulonephritis in the rat. *Journal of the American Society of Nephrology : JASN* 18, 1816-1823.

Takeuchi, K., Sereemasapun, A., Inagaki, T., Hakamata, Y., Kaneko, T., Murakami, T., Takahashi, M., Kobayashi, E., and Ookawara, S. (2003). Morphologic characterization of green fluorescent protein in embryonic, neonatal, and adult transgenic rats. *Anat Rec A Discov Mol Cell Evol Biol* 274, 883-886.

Tesson, L., Usal, C., Menoret, S., Leung, E., Niles, B.J., Remy, S., Santiago, Y., Vincent, A.I., Meng, X., Zhang, L., *et al.* (2011). Knockout rats generated by embryo microinjection of TALENs. *Nat Biotechnol* 29, 695-696.

van den Brandt, J., Wang, D., Kwon, S.H., Heinkelein, M., and Reichardt, H.M. (2004). Lentivirally generated eGFP-transgenic rats allow efficient cell tracking in vivo. *Genesis* 39, 94-99.

Whitelaw, C.B., Springbett, A.J., Webster, J., and Clark, J. (1993). The majority of G0 transgenic mice are derived from mosaic embryos. *Transgenic Res* 2, 29-32.

Will, C.C., Aird, F., and Redei, E.E. (2003). Selectively bred Wistar-Kyoto rats: an animal model of depression and hyper-responsiveness to antidepressants. *Molecular psychiatry* 8, 925-932.

Wolf, D., and Goff, S.P. (2009). Embryonic stem cells use ZFP809 to silence retroviral DNAs. *Nature* 458, 1201-1204.

Yamori, Y., and Okamoto, K. (1974). Spontaneous Hypertension in the Rat. In *Achtzigster Kongress* (J.F. Bergmann-Verlag), pp. 168-170.

Zheng, C., and Baum, B.J. (2005). Evaluation of viral and mammalian promoters for use in gene delivery to salivary glands. *Mol Ther* 12, 528-536.